MICROCOPY RESOLUTION TEST CHART

NATIONAL BUREAU OF STANDARDS 1963 A

Studies on Rickettsial Diseases (U)

ANNUAL PROGRESS REPORT

by

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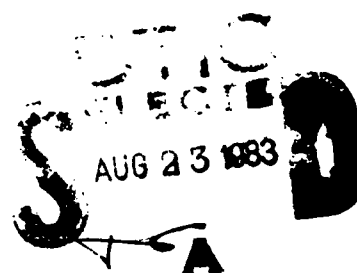
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SUMMARY

New methodology for the study of rickettsiae has opened the way for an enormous range of new studies of a quantitative nature. Most important among these is the routine application of modifications of the plaquing technique refined by Wike et al., the simple method for counting the absolute number of RLB described by Silberman and Fiset and various modifications of the tissue culture technique, including the slide chamber method. These methods have permitted the recognition of extraneous agents in most yolk sac seeds of rickettsiae, plaque-purification and cloning of rickettsial lines, definition of the intracellular growth cycle of Rickettsia prowazeki, investigations of host cell range of those rickettsiae and of the rickettsia-host cell interaction, methods for determining accurately the antibiotic sensitivity of rickettsiae. Reproducible, predictable in vitro systems appear to be possible.

Clinical data from Burundi has confirmed our original observation that a single 100 mg dose of doxycycline will cure louse-borne typhus and has established for the first time that minocycline is equally effective.

A field trial of the living attenuated E strain typhus vaccine in Bolivia confirmed its acceptability in this population.

FOREWARD

In conducting the research described in
this report, the investigators adhered to the
"Guide for Laboratory Animal Facilities and Care",
as promulgated by the Committee on the Guide for
Laboratory Animal Resources, National Academy of
Sciences - National Research Council.

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I. GENERAL

The development of newer technology in the study of rickettsiae has led to the progressive acquisition of much basic information and to the development of improved methods directly applicable to the solution of the practical mission-oriented problems of vaccines, chemotherapy, immunity, species identification, etc. Considerable effort has been expended under this contract to acquire the precise, quantitative methodology necessary for the solution of the practical problems which it encompasses and to apply them to the solution of those practical problems of military medical significance as rapidly as possible. Considerable progress has been made along these lines in the past year and the practical results, already beginning to emerge, will become increasingly more evident in the coming year.

II. RESEARCH RESULTS

A. Basic studies. In the past few years techniques for the
study of rickettsiae have improved immensely and within the past year
or so a sufficient variety of techniques have become reasonably well
established so that a totally new exciting era in rickettsiology has
been ushered in and it is now possible to approach basic problems of
the kind alluded to in the "Background" in a practical way never
before possible. Foremost among the techniques and methods have been
the following: (1) simple, rapid direct counting procedure for
rickettsial bodies in suspensions; (2) improved methods of purifica-
tion of rickettsiae, especially the sucrose batch technique (Wisseman
et al, to be published; this contract) for initial partial purifica-
tion and reduction of large volumes of crude suspension to small,
manageable volumes; (3) improved tissue culture techniques - plaque
method (23-29; this contract); slide chamber cultures (this contract -
see below) and roller bottle techniques (this contract); (4) fluores-
cent antibody methods; (5) application of electron microscopy to
rickettsial ultra-structure and immune mechanisms; and (6) increased
application of radioactive tracer techniques to various problems.
The development of some of these methods and their application to
current problems are described in sections which follow.

1. Development of methodology. In addition to establishing
the plaque technique, originally developed by others, as a routine
procedure in our laboratory and adapting it to several problems, we
have developed a slide chamber culture methodology which also has
proven extremely adaptable to different kinds of problems and has
provided a kind of quantitative information not otherwise obtainable.
Lab Tek^R chamber slides with 1-8 chambers have been used with non-
irradiated and irradiated cells, the latter providing non-multiplying
separated host cells suitable for counting rickettsiae. We have
adapted roller bottle cultures for the large scale production of cer-
tain rickettsiae and have demonstrated the feasibility of infecting
cells in suspension with rickettsiae for subsequent growth in cultures,
yielding highly uniform replicate cultures. These methods have been
adapted and applied to several of the studies described below.

2. Extraneous agents in rickettsial seeds and possible mix-
tures of rickettsiae from field isolates. New and more sensitive
methods often disclose problems not previously recognized. And so it
has been with the application of tissue culture methods, particularly
the plaque method in studies on antibiotics described below. Previous-
ly, when rickettsiae were simply passed in eggs for seed and production,
routine sterility checks occasionally showed bacterial growth and
these lots were excluded. It has been known for some time that fowl
leukosis viruses are present in probably all egg-grown rickettsial
preparations and these have been excluded from the E strain vaccine.

We initially found under the inhibitory influence of certain anti-
biotics which inhibit rickettsial growth, non-rickettsial plaque-
forming agents in low frequency. On two occasions, a bacterial agent
has been recovered in eggs from plaques and we suspect others are
caused by extraneous viral agents or mycoplasmas.

Further work, especially with egg-grown seeds of the spotted
fever group, both standard reference strains and recent field isolates
from Pakistan, has revealed in some instances great heterogeneity in
plaques, both in size and appearance, without the use of large inoc-
ula or selective agents. Control (uninoculated) flasks containing
cells from conventional (Truslow) eggs frequently produce spurious
plaques and even flasks containing cells from SPAFAS eggs occasionally
show a few spurious plaques. However, some seed preparations show
large numbers of plaques due to suspected extraneous agents, sometimes
exceeding the number of plaques thought to be of rickettsial origin.
Heating the serum used in the medium at 56C for 1-2 hr does not
reduce the number of spurious plaques.

We have tentatively considered the following as possible sources
of plaque heterogeneity;

    a. Rickettsial origin
       (1) Plaque size variants of a single species
       (2) Two or more different rickettsiae -- an
especially important consideration for field isolates derived from
pooled materials.

    b. Egg origin: bacterial, viral, mycoplasma
       (1) Agents in cells employed in plaque bottles.
       (2) Agents of egg origin in the seed material.
Most available seeds, even of reference strains, have been produced in
conventional eggs. It may be that extraneous agents have been pro-
pagated along with the rickettsiae for unknown numbers of passages.
The number of probable extraneous agents is so great relative to the
putative rickettsiae in some preparations that we have serious concern
over antigens which have been prepared from these and the serologic
results obtained as well as many other properties.

    c. Guinea pig, etc. Rickettsia-like agents have been
found in guinea pigs (Bozeman and Elisberg). Most of the rickettsial
strains in use have been passed at some time in guinea pigs. It is
possible that the seed lines could have been contaminated with such
agents from guinea pigs or with as yet unrecognized agents from other
animals used in passage.

      d. Original material from which agent was isolated - anthropod, mammal, etc. Unknown potential problem.

When we first encountered a few extraneous plaques under selective conditions, we thought that the simplest-solution to the problem would be to plaque-purify the rickettsiae and forget about the extraneous agents. Although we now plan to plaque purify all of our rickettsial strains, it is obvious that we must pay more attention to the plaque variants to (1) identify plaque variants of a single rickettsial species, (2) to isolate, purify and identify components of mixtures of rickettsiae and (3) to have methods available to detect readily the presence of the more common extraneous agents in seeds, antigens, etc. We must develop practical methods for preparing reliable, pure rickettsial seeds, antigens, etc. No studies are valid if mixtures are used: (It is nearing the century mark since Robert Koch introduced solid media for isolation of bacterial colonies!) Towards this end, we are evaluating cells which may be free of such agents in which rickettsiae can be propagated. Moreover, we are using the FA technique to verify plaque isolates as of rickettsial origin and are preparing antisera against the common extraneous agents so that these can be recognized in rickettsial preparations by either FA technique or other serological procedures.

Though laborious, frustrating and non-creative with respect to rickettsiae, this is absolutely necessary to assure improvements in rickettsiology and valid results from most kinds of studies.

      3. Growth cycle of R. prowazeki in chick embryo cell cultures.
We have adapted the slide chamber culture system with irradiated CE cells to the quantitative study of rickettsial uptake and intracellular growth by direct microscopic counting of rickettsiae in stained preparations. Though laborious and time-consuming, this method of approach has yielded a large amount of information not obtainable by indirect methods. A substantial study, which has provided an enormous amount of basic information applicable to other problems, is being prepared for publication. (See Figures 1 to 4 and Tables 1 and 2)

In summary, R. prowazeki is taken up during the infection period probably by active penetration into the non-phagocytic CE cells in a highly predictable manner following reasonably closely a Poisson distribution. Thus, by a single measurement -- the per cent cells infected -- we can describe the distribution of rickettsiae among the cells -- i.e., the per cent which have 1, 2, 3, 4, etc., rickettsiae. This becomes important in at least two situations: (1) too large an inoculum produces cytotoxicity to the host cells and causes cells to strip from glass or plastic. We are close to defining the

Figure 1

Time-Course of R. prowazeki (Breinl) Uptake by Non-Irradiated and Irradiated CE Cells in Stationary Slide Chamber Cultures
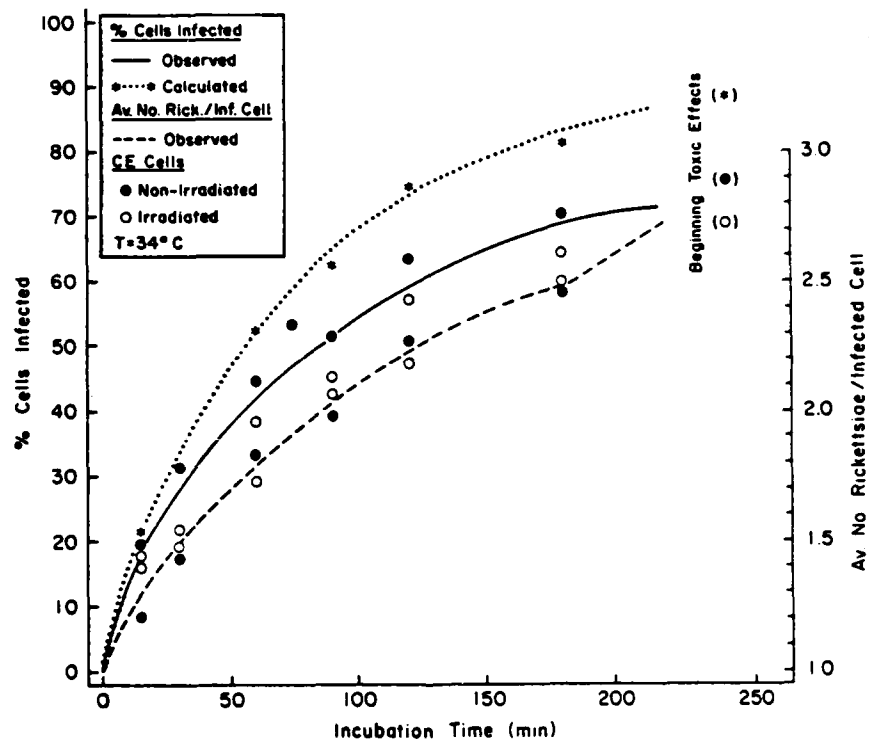
Figure 2

Uptake of R. prowazeki (Breinl) by Irradiated and Non-Irradiated
C E Cells in Slide Chamber Cultures as a Function of Rickettsial Concentration

Figure 3

"SINGLE STEP" GROWTH CYCLE OF *R prowazeki* (Breinl strain) IN SECONDARY IRRADIATED CHICK EMBRYO CELL CULTURES

Linear Regression Analysis (0-36 hr)

$\log_2 N = 0.114t + 1.328$ $r = 0.992$

$g = 8.8$ hr

$R = 0.114$

$K = 0.079$
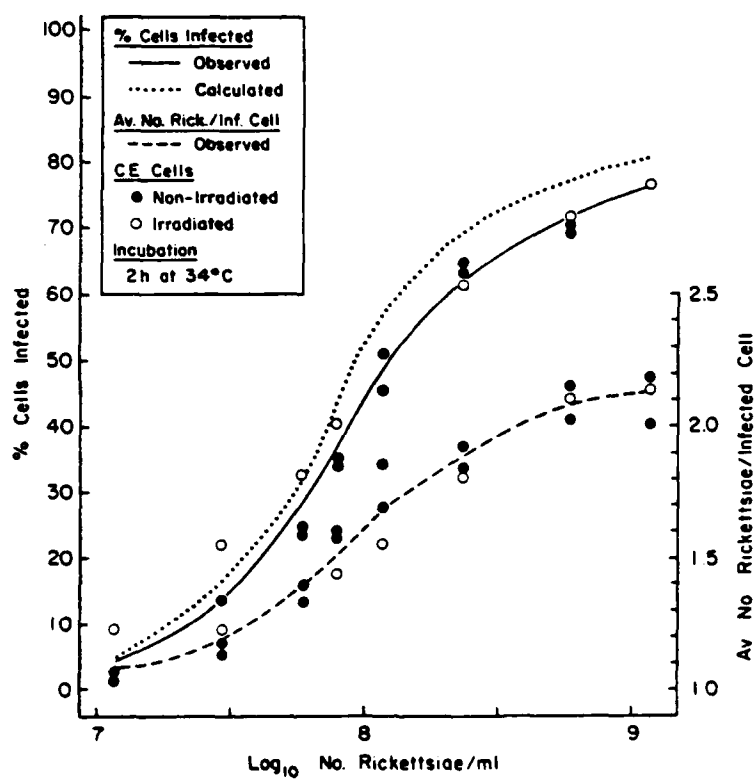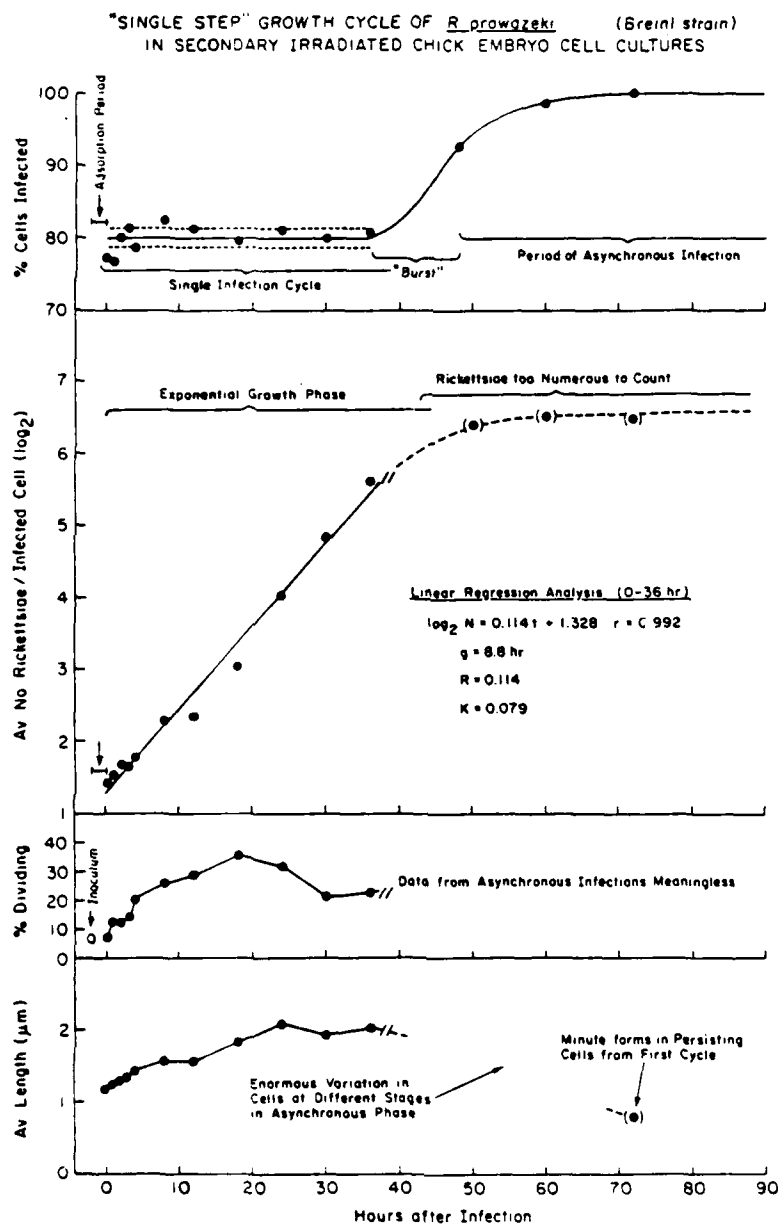
Figure 4

GROWTH CYCLE OF R prowazeki (Strain E) IN IRRADIATED CHICK EMBRYO FIBROBLASTS IN SLIDE CHAMBER CULTURES

R prowazeki, Strain E
Slide Chamber Cultures
Irradiated CE Fibroblasts
Dulbecco's Medium
T=34°C

"Burst"

Progressive Cell Loss

"Single Growth Cycle"

% Cells Infected

Cells too Loaded for Accurate Counting

Linear Regression Analysis (0-37 hr)

$\log_2 N = 0.112t + 0.846$

$g = 8.93 \, hr$ $r = 0.997$

$R = 0.112$

$k = 0.077$

Av No Rickettsiae per Infected Cell ($\log_2$)

Time after Infection (hr)

<center>Table 1</center>

Comparison of Observed Distribution of R. prowazeki (Breinl) in Irradiated CE Cells in Slide Chamber Cultures with Distribution Predicted from Per Cent Cells Infected $[(1-P)\times 100]$

Adsorption Time (min)		% Cells with r Rickettsiae					
		0	1	2	3	4	5
30	Obs	78.45	13.89	5.45	1.71	0.33	0.08
	Calc*	—	19.00	2.31	0.19	0.01	0.00
60	Obs	61.87	20.79	10.42	4.00	1.79	0.58
	Calc	—	29.70	7.13	1.14	0.14	0.01
120	Obs	42.83	27.69	14.32	6.54	3.12	2.04
	Calc	—	36.32	15.39	4.35	0.92	0.16

$*m = -\ln p \quad \%\,Cells_r = \dfrac{m^r e^{-m}}{r!} \times 100$

<center>Table 2</center>

Comparison of Observed Distribution of R. prowazeki (Breinl) among Irradiated CE Cells in Slide Chamber Cultures with Distribution Predicted from Average Number of Rickettsiae per Cell
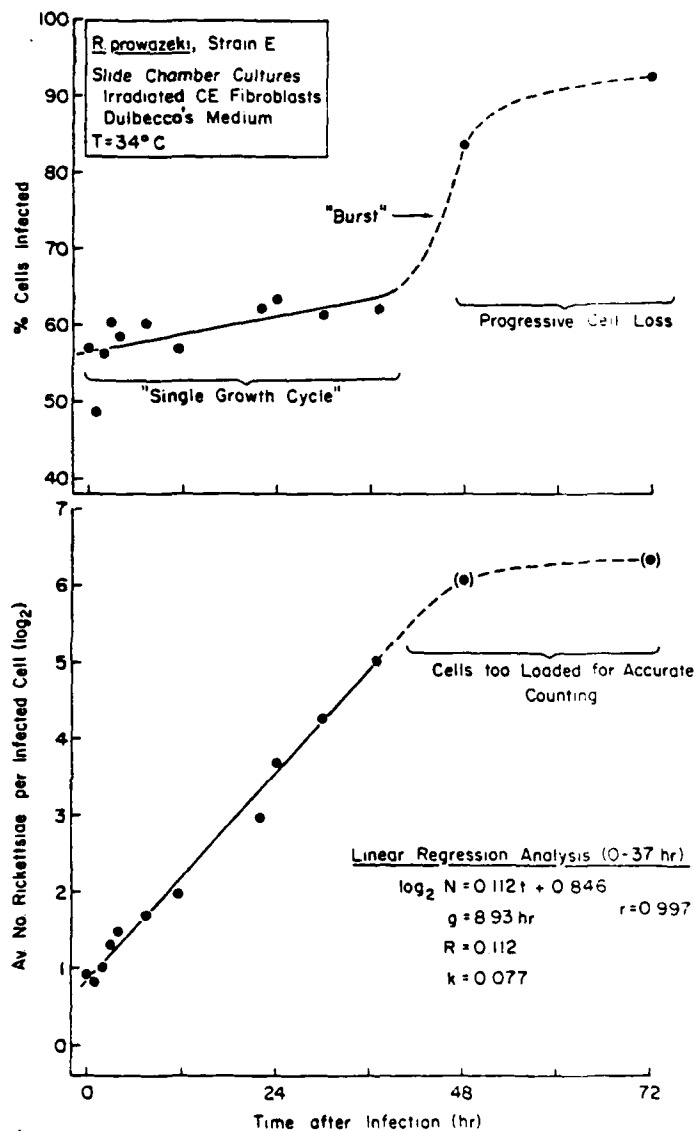
Adsorption Time (min)		% Cells with r Rickettsiae					
		0	1	2	3	4	5
30	Obs	97.9	2.1	0.0	0.0	0.0	0.0
	Calc*	98.0	2.0	0.0	0.0	0.0	0.0
60	Obs	80.4	17.6	1.6	0.4	0.0	0.0
	Calc	80.3	17.6	1.9	0.1	0.0	0.0
120	Obs	46.9	29.6	14.0	6.4	1.9	1.5
	Calc	39.5	36.7	17.1	5.3	1.2	0.2
180	Obs	41.6	25.4	17.3	8.7	3.4	2.7
	Calc	30.4	36.2	21.4	8.5	2.5	0.6

$*\,\%\,Cells_r = \dfrac{m^r e^{-m}}{r!} \times 100$

Table __3__ . Failure of Cytochalasin B to inhibit significantly uptake of R. prowazeki by CE cell monolayers as indicated by subsequent plaque production.

Cytochalasin B (μg/ml)	Uninoculated Cell Sheet	Plaques (% Control)
0	intact	100
5	intact	88.6
10	intact	87.7
20	lysed	- - - -

CE cell sheets in T-30 flasks were exposed at 24 C to cytochalasin B in TC medium for 15 minutes prior to inoculation and during the 30 min rickettsial adsorption period, after which cytochalasin B and unadsorbed rickettsiae were removed by 2 washes with growth medium. The cell sheets were then overlaid with agar and incubated in the usual manner for plaque formation.

number of rickettsial penetrations that produce irregular host cell
membrane damage. From this we can determine the optimal degree of
infection for maximum rickettsial yields. (2) In genetic studies
searching for evidence of recombination, we can reliably predict the
proportion of host cells which will contain 2 or more rickettsiae
under conditions where genetic interaction might be possible. More-
over, we now also have a mathematical or statistical basis for
quantitating the influence of variables on the penetration of host
cells by typhus rickettsiae.

Once within the CE cells in the system employed, R. prowazeki
begins to grow and multiply by binary fission without detectable lag,
in contrast to the findings of Kordova in L-cells which probably are
hostile, and they enter almost immediately into an exponential or log
growth phase with a generation time of 8-9 hrs. There is no evidence
for an "eclipse" phase postulated by Kordova. The exponential growth
continues until the intra-cellular rickettsiae are so numerous that
they can no longer be counted accurately. It is unknown at this time
if they enter into a stationary phase, a matter which will be clari-
fied in another system. However, they go through changes in size and
morphology typical of bacteria in fluid culture and the organisms in
loaded and deteriorating host cells are very small in comparison with
those in early log phase growth. It is reasonable to expect the
rickettsiae to undergo physiologic and antigenic changes in the growth
cycle just as other bacteria do, for the rickettsiae behave in an
infected CE cell just as a bacterium might in a tiny broth culture!
(See Figure 3).

The duration of the infection cycle, i.e., from time of uptake
to final breakdown of the host cell and release of the rickettsiae,
in this system is between 36 and 48 hrs. When the CE cells in culture
are infected within a short time, the infection in the entire culture
progresses in the same manner. While we cannot claim true synchrony,
we are approaching an ideal one step growth cycle. The "burst" phase
is ragged, occurring over a good many hours so that the second cycle
is grossly asynchronous. However, if a high proportion of cells are
infected initially, then substantial yields of rickettsiae in any
predetermined phase of growth should be possible. It should then be
possible to ascertain if important antigenic or other changes take
place during the growth cycle and to devise means to produce rickettsiae
with uniform properties. This has obvious direct application to the
problems of killed vaccines and diagnostic antigens.

A similar study was performed with the attenuated E strain of Rickettsia prowazeki with almost identical results. The generation time if the E and Breinl strains on CE cells were the same within limits of experimental error. Thus, in this cell system the attenuated E strain behaves in a manner indistinguishable from that of the virulent Breinl strain, (See Figure 4).

We have not yet had time to study the influence of the medium composition on rickettsial growth but predict that it will be substantial. We have demonstrated that CE cells in suspension can be infected for the preparation of highly uniform replicate cultures but we must still work out optimum infection load, etc. Nevertheless, this has already been applied to the preparation of slides for indirect fluorescent antibody tests in which the rickettsiae were in active log phase growth and presumably bore optimum amounts of antigenic components. (Rickettsiae from asynchronous cell cultures in the past have been variable and not very satisfactory for FA tests!)

We have now begun to scale up the experiments to stationary flasks and roller bottles to (1) complete the growth studies to include measurements of biological function and immunogenicity and (2) develop practical production methods for rickettsiae in defined growth states.

4. Host cell range -- permissive, non-permissive and discriminating cells. It has become clear that the fate of R. prowazeki is different in different kinds of host cells -- i.e., the fate of the rickettsiae depends on properties of the host cell they enter. This phenomenon can be used to advantage towards the solution of certain applied and basic problems if the right cells are found. In parallel experiments with the virulent Breinl and attenuated E strains of R. prowazeki, we have found that chick embryo cells and SV-40 transformed continuous mouse peritoneal macrophages permit equal growth with both rickettsial strains and that fresh mouse peritoneal macrophages do not permit either strain to grow, but that human peripheral monocytes allowed to develop into macrophages in cell culture permit the virulent strain to grow but either destroy or do not permit the attenuated E strain to grow. (See section on virulence below.) Moreover, neither CE cells nor transformed mouse peritoneal macrophages will destroy antibody-sensitized virulent R. prowazeki but the human macrophages will destroy them.

The objective here has been to find cells which will do the following, preferably also yielding plaques:

a) Yield maximum numbers of virulent and attenuated rickettsiae for production of rickettsiae in cell culture and permit isolation and quantitation of rickettsiae regardless of virulence.

CE cells will do this and will form plaques.

b) Permit antibody-sensitized rickettsiae to grow and be quantitated, so that rickettsiae can be isolated and counted from immune hosts or systems. CE cells will do this. (See Figure 5).

c) Kill antibody-sensitized rickettsiae, to permit the development of an in vitro neutralization test which detects protective antibody. Human macrophages do this, but are not easily adapted to routine tests.

d) Discriminate between virulent and attenuated R. prowazeki. Human macrophages do this, but plaquing for quantitation of virulent back-mutations is not yet possible with these cells.

To date we have found that fresh mouse peritoneal macrophages do not permit growth of either the Breinl or E strains and that the following cells permit both the Breinl and E strains, as well as antibody-sensitized Breinl strain to grow in much the same manner that the CE cells did: SV-40 transformed continuous line of mouse peritoneal macrophages, HeLa, KB and L. (See Figure 6).

Thus, while we have cell types which will achieve certain of the objectives in practical or routine type situations and we have a cell type (human macrophage) which will accomplish the other objectives in principle, demonstrating them as reasonable objectives, we must find cells which will perform in the desired manner, which are practical to produce in quantities for routine use and which will yield plaques so that the laborious and time-consuming microscopic examination can be eliminated. The continued search for such cells is a part of the future plans. With the complete array of cells described above, we can attack directly several of the important outstanding problems of vaccines, both killed and living attenuated. At the same time, the results also contribute to a basic understanding of host cell-rickettsia interactions.

5. Antibiotics. An in vitro study of selected antibiotics and R. prowazeki was undertaken with the following objectives: (1) to develop practical, precise methods for the measurement of the sensitivity (MIC) of rickettsiae to growth inhibition (rickettsiastasis) by antibiotics; (2) to develop practical, precise methods for the detection and quantitation of rickettsiacidal action of antibiotics; (3) to develop practical methods for the detection and quantitation of antibiotic resistant rickettsial mutants; (4) to ascertain if the dramatic therapeutic effect of doxycycline and minocycline in louse-borne typhus (see section on therapy below) is due to some unusual action upon the rickettsia or if it is merely a function of prolonged blood and tissue levels; (5) to identify possible antibiotics for treatment of disease as alternatives to the tetracyclines and

EFFECT OF PRE-TREATMENT WITH NHS, IHS OR MBSA ON FATE OF R. prowazeki IN IRRADIATED
CE CELLS IN SLIDE CHAMBER CULTURES

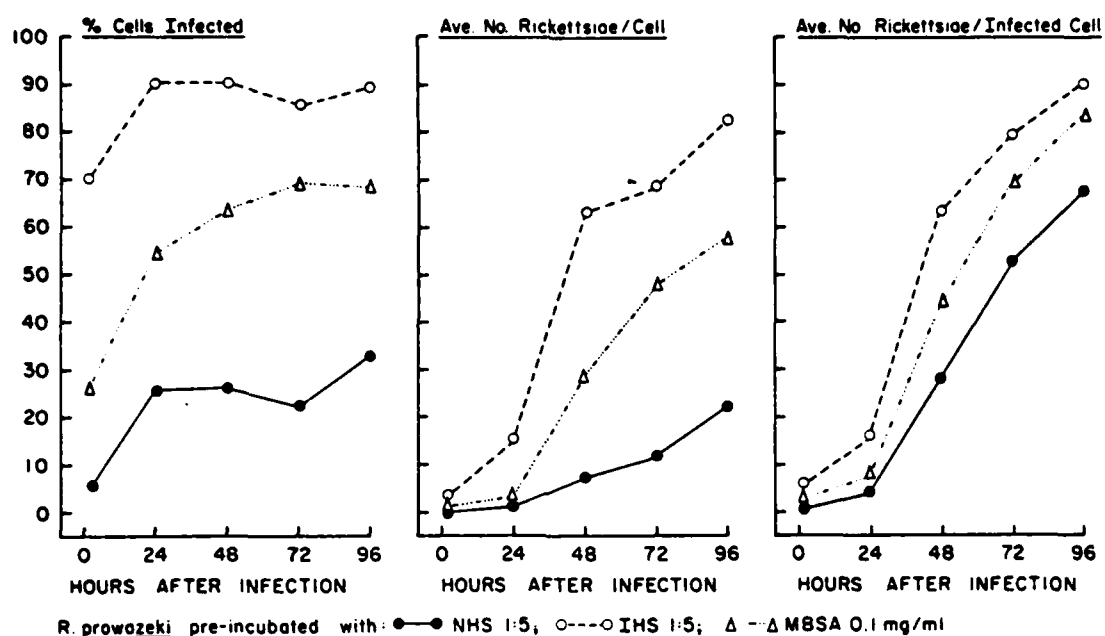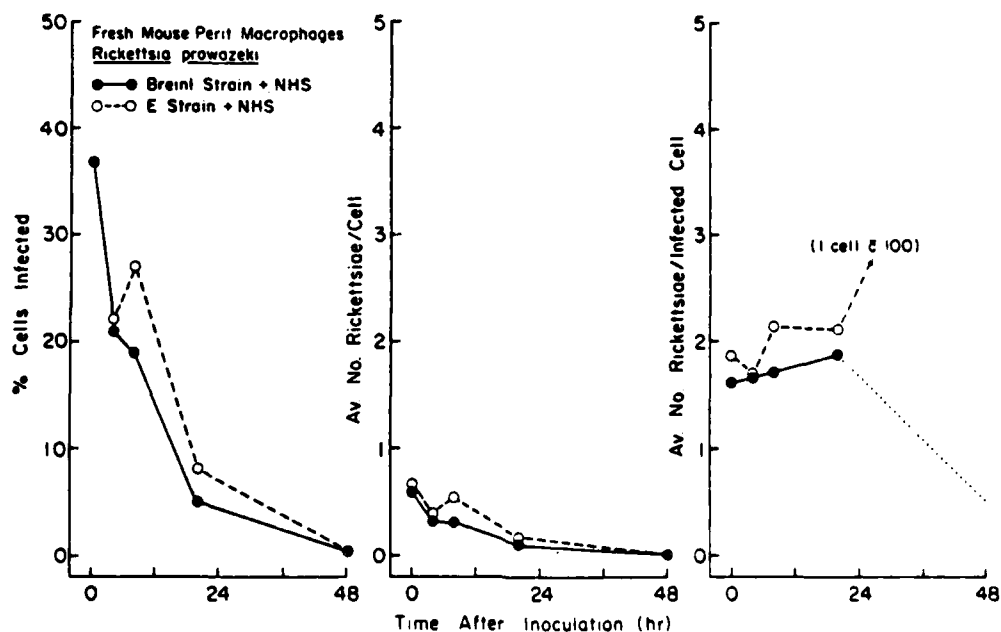

R. prowazeki pre-incubated with: ●——● NHS 1:5, ○---○ IHS 1:5; △——△ MBSA 0.1 mg/ml

Figure 6

Fate of Virulent and Attenuated R. prowazeki Strains in Fresh (48 hr) Mouse
Peritoneal Macrophages in Slide Chamber Cultures

chloramphenicol in the event that a resistance problem is encountered;
and (6) to find antibiotics which can be used to inhibit bacterial
growth in rickettsial cell cultures.

The two basic methods described in "a" above, i.e., the slide
chamber culture method and the plaque method have been adapted very
satisfactorily to this problem. Each method yields different kinds
of important information, but both methods yield comparable values
for minimal inhibitory concentration. A plaque reduction method
yields very precise information about minimal inhibitory concentration.
A delayed overlay technique has been devised to measure in precise
quantitative terms the rate at which an antibiotic kills the
rickettsiae.

Results to date are summarized as follows. (See Figures 7 to 12).
(1) The minimal inhibitory concentrations of selected antibiotics
for virulent R. prowazeki are penicillin G (20-50 µg/ml, chloramphenicol
(1.0 µg/ml), tetracycline, doxycycline, minocycline (0.1 µg/ml),
erythromycin (0.06 µg/ml) and rifampin (0.01 µg/ml). At a clinically
attainable concentration with usual therapeutic doses (1.0 µg/ml),
doxycycline, rifampin and erythromycin each exerted a definite, but
slow rickettsiacidal action. The rate of killing, however, was too
slow to suggest that eradication of the organism from the body could be
achieved under the usual practical therapeutic situation. Hence, none
offers a practical solution to the human reservoir problem.

Penicillin induces spheroplast formation by R. prowazeki within
infected cells and rapidly kills a substantial fraction of the organisms
within 24 hr, leaving a penicillin-insensitive residual fraction. If,
as these studies in progress suggest, the spheroplasts are osmotically
unstable within host cells, we may have a mechanism for releasing
rickettsial DNA within the host cell to facilitate genetic interaction
between strains. Moreover, some penicillin or cephalosporin derivative
may produce similar lethal effects at a practical therapeutic concentra-
tion, i.e., a practical rickettsiacidal antibiotic. (See Figures 13-15).

We have been reasonably successful in developing methods for the
quantitation of mutation to antibiotic resistance. If mutation rates
and frequencies to antibiotic resistance are similar to those of other
bacteria, a large rickettsial inoculum into a plaque bottle under
selective conditions will be necessary. The practical problem is that
excessively large rickettsial inocula, even in the presence of many
antibiotics, have a cytotoxic effect and cause stripping or lysis of the
CE cell monolayer. We have now got to the point where we can success-
fully introduce 10^7 PFU of R. prowazeki into a single T-30 flask
without irreparably damaging the CE cell monolayer. We

Figure 7
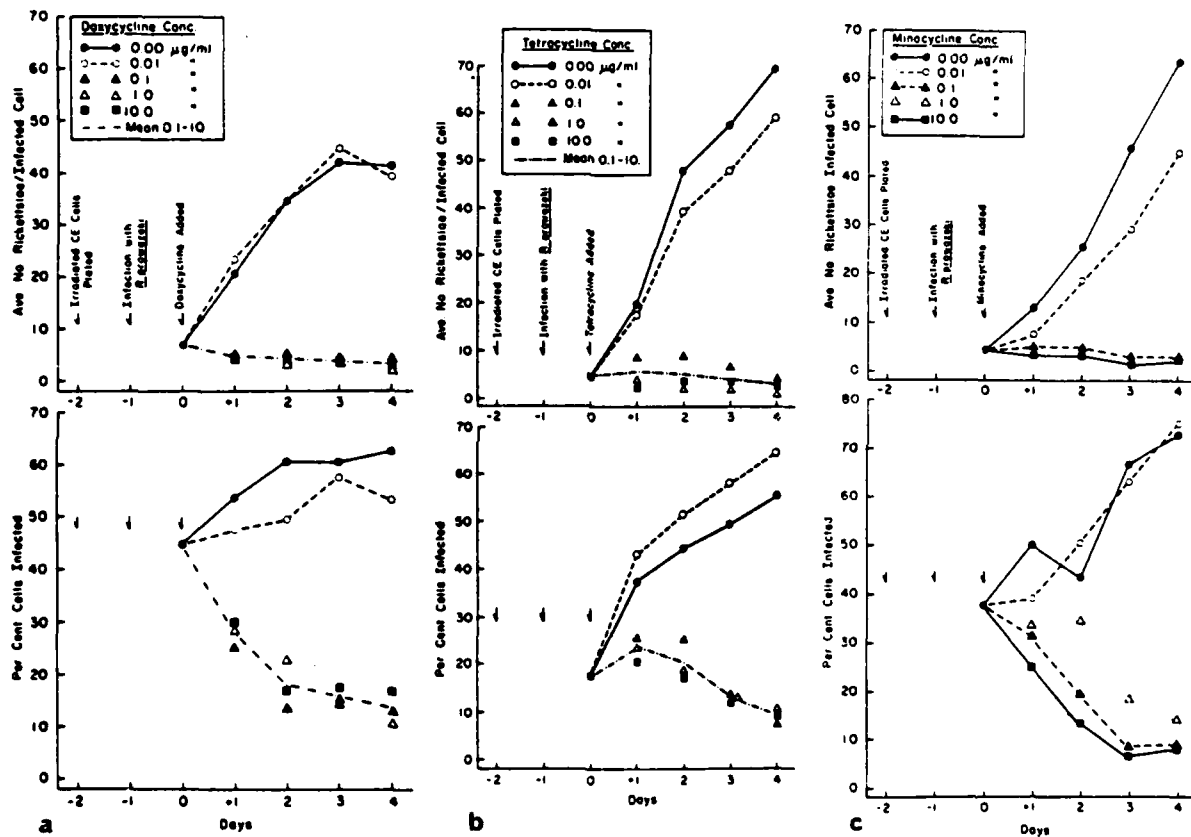
Figure 8

RICKETTSIASTATIC AND RICKETTSIACIDAL ACTION OF RIFAMPIN
ON R. prowozeki BY SLIDE CHAMBER CULTURE METHOD
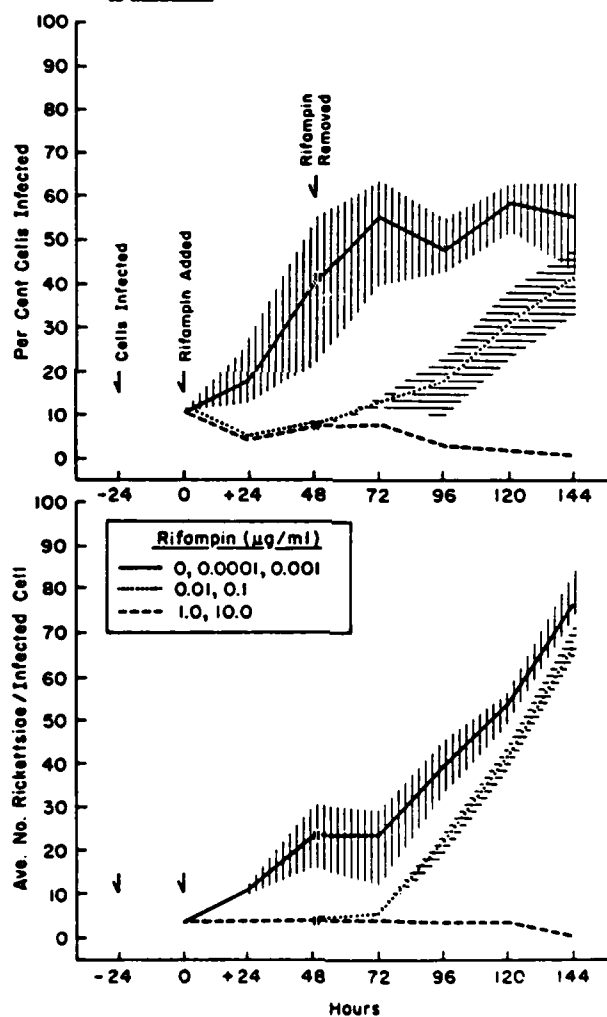
Figure 9

DOSE-RESPONSE CURVES OF R. prowazeki (Breinl Strain) TO
VARIOUS ANTIBIOTICS BY PLAQUE REDUCTION METHOD

●—● Rifampin   ●---● Penicillin G
○—○ Doxycycline   △·····△ Chloramphenicol
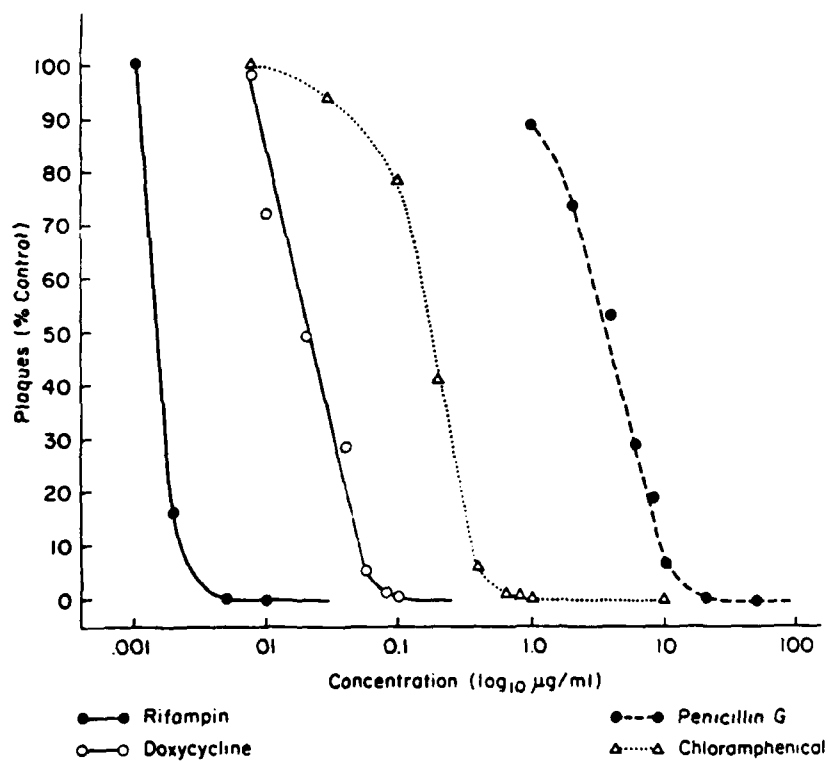
Figure 10

DOSE-RESPONSE CURVES OF THE E AND BREINL STRAINS OF
R. prowazeki TO ERYTHROMYCIN BY PLAQUE REDUCTION METHOD
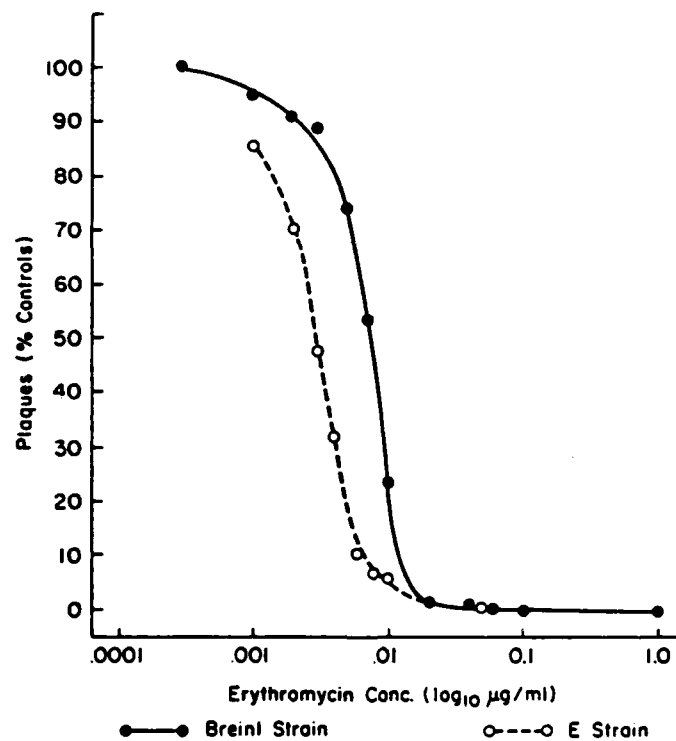
Figure 11

RICKETTSIACIDAL ACTION OF ANTIBIOTICS ON R. prowazeki
(Breinl) BY DELAYED OVERLAY PLAQUE REDUCTION METHOD

Key	Drug	Conc. (μg/ml)	50% Kill
●—●	Rifampin	1	9.8 hr
○--○	Doxycycline	1	22.1 hr

Plaques (log₁₀ % Control)

Exposure Time to Antibiotic (hr)
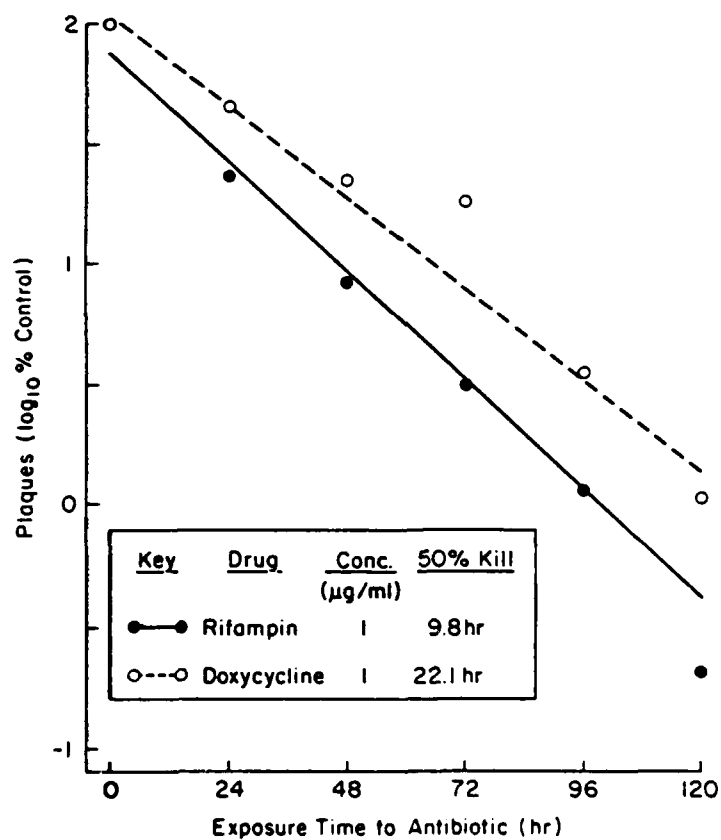
Figure 12

RICKETTSIACIDAL ACTION OF ERYTHROMYCIN ON
R. prowazeki (Breinl) BY DELAYED OVERLAY PLAQUE
REDUCTION METHOD

Erythromycin
1 μg/ml

Plaques (log₁₀ % Control)

Exposure Time to Erythromycin (hr)
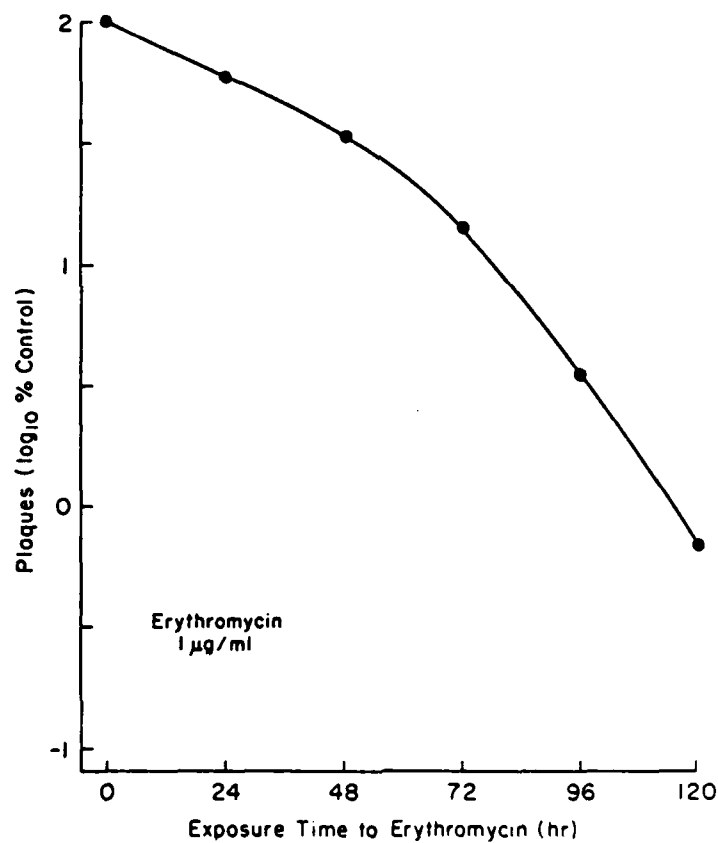
Figure 13

Inhibition of Plaque Formation by Penicillin G
on R. prowazeki in CE Cell Cultures
by Delayed Overlay Method

Figure 14

Penicillin-Induced Unstable Spheroplast Formation
in CE Cells Lightly Infected with *R. prowazeki*
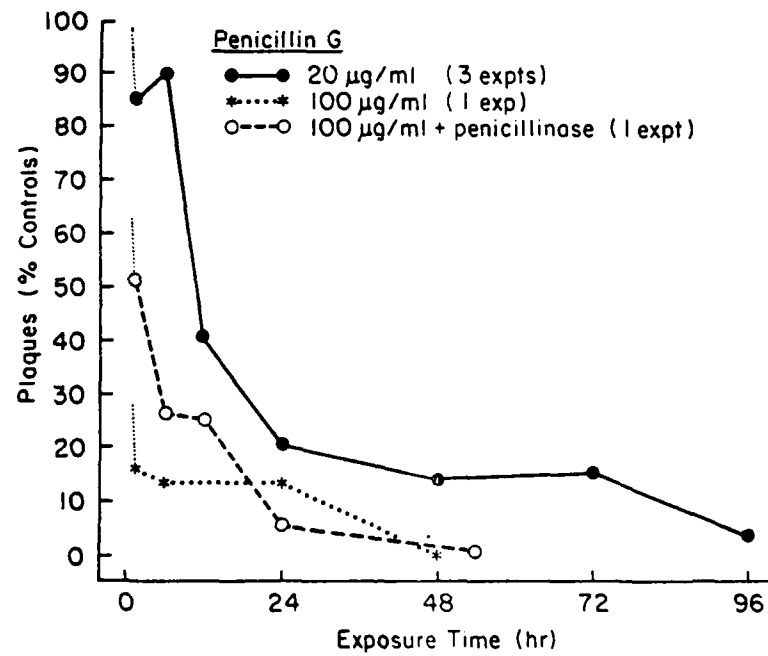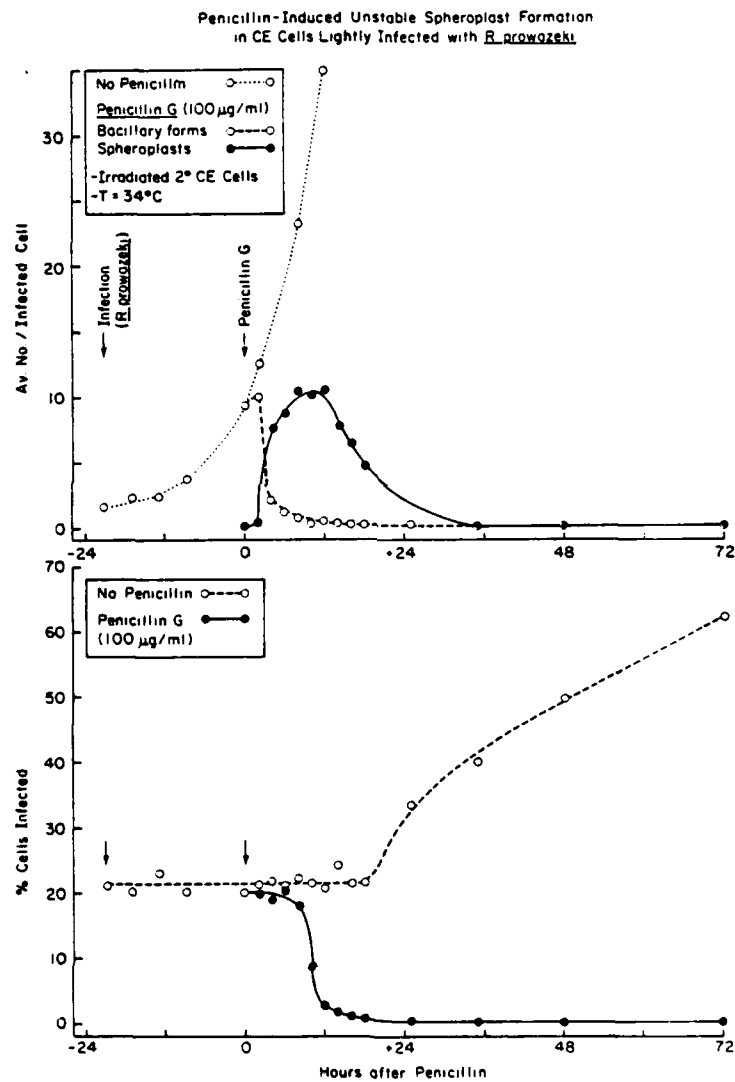
Figure 15

Penicillin-Induced Spheroplast Formation Followed by Host Cell Destruction in CE Cells Heavily Infected with *R. prowazeki*
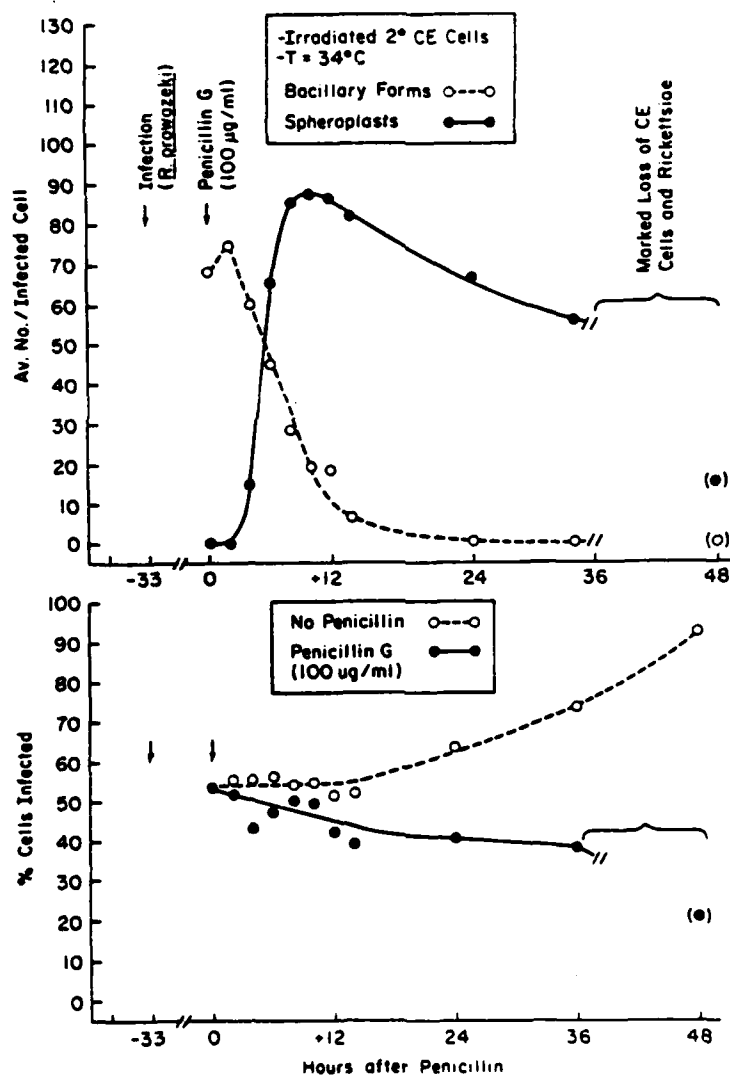
have already had some limited success in measuring resistant mutant frequencies with chloramphenicol and rifampin. But these are also the conditions under which "plaques" by antibiotic insensitive extraneous agents, referred to in "b" above, begin to interfere significantly with quantitation. This problem will be eliminated when seed stocks freed of these agents have been prepared.

6. **Immunity.** The role of humoral antibodies and their participation with professional phagocytic host cells in immunity is beginning to unfold into a major immune mechanism in typhus, making it reasonable to examine serum for certain kinds of antibody as a measure of immunity or as an immunogenic potency assay for vaccines. Very briefly, human convalescent typhus serum confers strong passive protection to animals against virulent challenge, opsonizes the rickettsiae for enhanced ingestion by macrophages and polymorphonuclear leukocytes, but has no direct rickettsiacidal action. Human macrophages alone do not restrict the growth of virulent R. prowazeki, since these organisms rapidly escape from phagocytic vacuoles to grow free in the cytoplasm, but antibody-sensitized rickettsiae are retained in phagocytic vacuoles where they are destroyed, presumably by lysosomal enzymes. Non-specific opsonization does not lead to destruction in these cells. On the other hand, the yolk sac cells of embryonated eggs, the midgut cells of the body louse, chick embryo fibroblasts in cell culture and continuous transformed mouse peritoneal macrophages are incapable of destroying antibody-sensitized R. prowazeki and permit unrestricted growth. Typhus specific antibodies cytophilic for human macrophages are present in convalescent serum, cause rosette formation with rickettsiae and enhance ingestion but probably do not lead to intravacuolar destruction. We believe that this system is complex and involves at least 2 rickettsial factors according to the following hypothesis; (1) surface antigens in the microcapsule which are somewhat antiphagocytic. Antibodies specific for this antigen(s) (at least 7 immunoprecipitin bands have been identified in typhus "soluble" antigen) opsonize and enhance phagocytosis but do not necessarily lead to intravacuolar destruction. (2) A rickettsial factor involved in cell penetration, escape from macrophage vacuoles and hemolysis. Antibodies against this factor, if they occur, would prevent escape from the macrophage vacuole and thus facilitate intravacuolar retention and destruction by lysosomal enzymes. We have a strong indication of the nature of this factor and how to extract it but have not yet specifically identified it. This is a very important project for the coming year because it may lead to identification of the "protective" antigen, means for detecting it and antibody response to it and to the development of meaningful potency assays for killed vaccines.

To date, of the cells tested, only human macrophages seem to permit virulent typhus rickettsiae to grow but destroy antibody sensitized rickettsiae. Fresh mouse peritoneal macrophages do not permit virulent R. prowazeki to grow in the absence of antibody (See Figure 6) whereas a continous line of SV-40 transformed mouse peritoneal macrophages permit both sensitized and unsensitized organisms to grow. The same holds for chick embryo cells in culture. (See Figure 5 and Table 3).

Although we know that delayed hypersensitivity develops as a result of typhus infection, we do not yet know the contribution of the T-cell mediated immunity to protection against disease.

7. Virulence. The importance of acquiring quantitative information on reversion to virulence by the E strain typhus vaccine is obvious. Virulent and the attenuated strains of R. prowazeki have been compared within a variety of parameters in the search for the basis for attenuation or, conversely, virulence. Electron microscopy of freeze-fractured and etched preparations show no difference between virulent and attenuated strains in thickness of the microcapsule, nor are there demonstrable serological differences, suggesting that it is not a simple smooth → rough variation. On a per organism basis, the virulent and attenuated strains show no essential difference in capacity to form plaques in CE cell monolayers, to hemolyze susceptible erythrocytes, to kill mice by toxic action, to grow in the midgut cells of body lice and to kill body lice. The generation time and growth rate in CE cells in cell culture for the two strains are within limits of experimental error. Our findings reported here differ somewhat from those of some earlier investigators who did not have the advantage of the techniques for quantitating organisms that we have developed. On the other hand, the two strains do differ in their capacity to infect mice inoculated i.p. and adult guinea pigs. A striking difference, however, is demonstrable at the cellular level with human macrophages in cell culture. The virulent strain survives, grows and kills the macrophage whereas the attenuated strain is destroyed. This observation is consistent with reduced virulence in man and laboratory mammals, where the professional phagocyte is a first line defense mechanism even without antibodies. As pointed out in "d" above, this finding establishes the feasibility of an in vitro virulence test which would lend itself to quantitation of mutations and reversions involving virulence and attenuation. We are concentrating on finding the appropriate cell system which will permit such studies to be made by simple, routine methods.

Again, the human macrophage in culture appears to have unique properties of discriminating between virulent and attenuated R. prowazeki. The fresh mouse peritoneal macrophage does not permit the growth of either virulent or attenuated strains (See Figure 6) whereas the continuous SV-40 transformed line permits both to grow, as does the chick embryo cell (Figures 3 and 4).

8. **Genetics of R. prowazeki.** Methods for the study of anti-biotic resistance (section "e" above) and virulence (section "g" above) have already been presented. With two independent genetic markers and appropriate indicator systems, the possibility of being able to induce DNA liberation from rickettsiae within host cells (section "e" above) and the statistical basis for quantitating the proportion of cells containing 2 or more rickettsiae, it is obvious that the methodology is nearly at hand to elucidate basic genetic mechanisms in R. prowazeki, using as marker characteristics of prime practical importance. We propose to bring these studies to a practical level of function.

9. **Miscellaneous.** We have demonstrated the applicability of the plaque technique for the detection, quantitation and isolation of R. prowazeki and R. mooseri directly from the tissues of infected guinea pigs and human body lice (Pediculus humanus humanus), respectively.

B. **Chemotherapy of louse-borne typhus.** By very good fortune, despite the civil strife in Burundi, we have been able to continue our studies on the chemotherapy of louse-borne typhus at the dispensary of the Mission de Katara where we conducted our field trials in 1969. The results of this study, which continue to be extremely rewarding, can be stated simply: (1) In over 300 cases of typhus, most with adequate serological confirmation of diagnosis, a single 100 mg oral dose of doxycycline cures most adults and a single 50 mg dose cures most children. Relapses are rare and, when they occur, they respond to a second dose of doxycycline. The doxycycline series has just about been completed. (2) A single 100 mg oral dose of minocycline, another "long-acting" tetracycline derivative, also cures most adult typhus patients and, in about 50 confirmed typhus patients to date, appears equally as effective as doxycycline. This series is continuing to build up larger numbers. (3) Cortisone and hydro-cortisone continue to serve as useful adjuncts to antimicrobial therapy in the comatose patient, the severely ill patient or the patient with severe dysphagia. (4) In very severely ill patients, anti-microbial therapy, even with adjunct cortico-steroid therapy, may not prevent death, even though the temperature response to antibiotic is adequate. This has been emphasized recently in 3 cases which died despite these two measures. Other supportive measures were not available in this "bush" facility, but it is by no means certain that even the most sophisticated hospital could have prevented this. Because of the recent civil strife, people have been extremely reluctant to come out of hiding and sometimes wait to seek medical aid until they are essentially moribund. To date, the number of such cases has been small -- 3 in a recently received set of observations. However, in those that survived for more than 24 hrs, the response of the fever to therapy was as expected, suggesting that these were not anti-microbial drug failures, which might have indicated antibiotic resistance. (Note: most of the fatalities due to RMSF in this country, even in good

Hospital, can be traced to delay in giving specific antimicrobial therapy.)
Rather, they are regarded as the result of the advanced stage of vascular and
tissue damage already existing that is not directly affected by antimicrobial
agents.

On the basis of the in vitro tests described above, it was postulated that
both erythromycin and rifampin should have a good therapeutic effect on louse-
borne typhus. So far, three patients have been treated with conventional oral doses
of each with a very satisfactory response. Hence, two new class os drugs can be
used for the therapy of louse-borne typhus, should tetracycline resistant strains
of R. prowazeki be encountered. (See appendix 1 for discussion of potential anti-
biotic resistance problem in nature.)

C. Field studies on the attenuated E strain vaccine.
Observations on the field trial of the E strain vaccine initiated in Burundi in
1969 in the face of a large epidemic are no longer feasible. Identification of
vaccinees had already become extremely difficult between 2 and 3 years after vaccin-
ation and the recent civil strife in that country made further efforts useless.
Typhus is still occurring in Burundi, though at a lower rate, even in the general
region of the vaccine trial which was very active in 1969. A final report of this
successful trial, which demonstrated good portection against disease during the
height of the epidemic, will be prepared. Some attempts are still being made to
identify precisely certain persons who claimed to have participated in the controlled
trial.

The initial field trial in Bolivia, an endemic typhus zone, is drawing to a
close. A set of serum specimens, representing a sample of the population of one
study area taken one year after vaccination has just been received. The results
of the serologic tests on these specimens will constitute the final set of obser-
vations in this trial. The first post-vaccination set of specimens, taken 6 wks after
vaccination, clearly demonstrated the feasibility of altering the immunologic profile
in an endemic stiuation in a predictable way which would be expected to interrupt
the continuous, low-level transmission which sustains the endemicity.

The final, 1 + yr, serum sample from this first pilot study in Bolivia
has finally been received in Baltimore and was tested for persistence of CF
antibody just recently. Although there had been a demonstrable decline in overall
titer, a not unexpected finding on the basis of earlier work by Fox et al., the
general shape of the age-specific distribution of antibodies was maintained. Since
CF antibody titer declines more rapidly than TN titer after E strain vaccination and
since protection against challenge was most closely correlated with TN titer, these
results suggest that the vaccine-induced resistance profile also persisted for
more than a year. A final report, summarizing the complete study, will be pre-
sented in a subsequent report.

Another population of about 8000 persons near Lake Titicaca has been
identified and characterized by Bolivian health officials for a second small-
scale field trial, which will be a controlled trial under very close observation for
the 3 weeks after vaccination to assess reactions and acceptability in quantitative
terms. The first trial, while giving much information about antibody responses in an

endemic situation, yielded only a general impression of acceptability
and was lacking in quantitative measures of reaction rates -- for
both practical and political reasons. This second pilot study will be
a controlled trial, with equal numbers randomly selected receiving E
strain typhus vaccine and tetanus-diptheria toxoid. A team of
physicians and ancillary personnel has been arranged specifically
to evaluate the reactions. The new CRD-3 lot of vaccine will be used
for the first time. Thus, an evaluation of the vaccine stock for
military use will be obtained as well.

D. Spotted Fever Group Rickettsiae. The mission of this project
has been to complete the identification of the tick-borne spotted fever
group isolates from Pakistan and Thailand, to describe the new species
which are represented, to clarify the variation observed between two
relatively recent Czech. R. sibirica-like isolates and some Pakistani
R. sibirica-like isolates with Soviet strains of R. sibirica and cer-
tain other Pakistan isolates, and to ascertain if the one Israeli
isolate in our possession (of 2 known isolates which are said by
Israeli scientists to differ from R. conori and from each other) is in-
deed another new species.

Our previous studies based on cross-toxin neutralization tests had
indicated that: one of the Pakistani isolates was similar to R. conori;
several were similar to R. sibirica but that these strains fell into
2 sub-groups, one closely related to Soviet strains of R. sibirica and
the other resembling 2 strains isolated in Czechoslovakia; and that
five strains, closely related to one another, probably constituted a
new species. Moreover, the single Thai isolate obtained from Elisberg
appeared to be still another new species.

The basis for speciation among strains of the spotted fever group
is poorly defined. Most of the strains now recognized as species were
geographically separated from one another and were described as species
on the basis of limited information. Only two critical studies, aside
from the current study, have ever been performed comparing the then
accepted strains or species - one at the Rocky Mountain Laboratory
using the toxin neutralization test and the other by Bozeman and Elis-
berg at WRAIR using the vaccination-challenge method. Most of the
species had originally been described on the basis of much less critical
testing. The toxin neutralization test and the vaccination-challenge
test are laborious, time-consuming and difficult. Toxins are especially
difficult to prepare. Moreover, unless a strain produces well-defined
illness in guinea pigs, it is difficult to carry out a satisfactory
vaccination-challenge test.

New, simple and highly specific tests are sorely needed and the
results of various tests must be compared in order to assess their
reliability, consistency, etc. with regard to speciation. We hesitate
to publish new species names without establishing a better basis for
speciation than exists. Yet, if the putative new species are valid,
we will have introduced important new information about the heterogeneity
of immunologic types in the spotted fever group and new information on
distribution and zoogeography. Moreover, since killed vaccines with
members of this group have tended to induce species-specific immunity,
with limited cross-immunity, knowledge of the number of species and
their distribution within an area is of considerable importance to
military preventive medicine policies.

Others have suggested from time to time that two relatively
simple serological procedures exhibit **reasonable** specificity -- the
microagglutination test of Fiset _et al_ and the fluorescent antibody
inhibition test. The latter has been the main method used by Israeli
scientists to differentiate the two putative new species from R. conori
which also exists in the area. Others have reported using the CF test
with specific rickettsial body antigens. We are tooling up to compare
all of these methods.

      1.   <u>Antigen production.</u> Production of antigens in suitable
quantity and of practical quality has plagued the study of spotted
fever group rickettsiae since the yields are very poor and the quality
is often unsatisfactory. We have addressed ourselves to this problem
in the past year.

      a.   <u>Rickettsial body antigens for microagglutination
and CF tests.</u> During the past year we have grown various members of
the spotted fever group in the yolk sac of embryonated hen's eggs and
now have either purified or concentrated, partially purified preparations
for most of the prototype reference strains and representatives of the
field isolates. Preliminary tests indicate that the purified antigens
form acceptable sedimentation patterns for the microagglutination test.
Their specificity remains to be demonstrated. See below for CF test.

      b.   <u>Antigens of defined growth state for FA tests.</u>
Making use of the basic information on the growth cycle of typhus
rickettsiae in the basic studies, we have now devised methods by which
chick embryo cells can be infected with spotted fever group rickettsiae
in suspension, the organisms grown to a mid-log phase when they are
numerous enough to be seem easily and are still in full physiologic
optimal state, transferred to special FA slides where the cells will
attach and flatten and then fix for subsequent use in the FA test.
This method is capable of producing large numbers of slides of highly
uniform characteristic, with organisms in optimal growth phase when
they should have their full complement of antigens and without losing
surface antigens since the organisms have never been manipulated out-
side of their host cell. Preliminary studies suggest that these
preparations will serve superbly as antigens for the FA test.

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This method is far superior to the use of infected yolk sacs in which the stage of growth cannot be carefully regulated. Consultation with Israeli scientists, who developed the FA method with yolk sac antigen, revealed that only one in many yolk sacs, a matter of pure chance, would serve as a satisfactory antigen source. We believe that we have now developed the technology to the point where we can eliminate this kind of variability and uncertainty.

2. <u>Extraneous agents in antigens</u>. After we had prepared most of the antigens described above, our tissue culture technology developed to the point where we could examine the seed preparations for extraneous agents, plaque variants and possible mixtures of rickettsiae by the plaque technique. This has been described in a preceding section. As pointed out in that section, some of the spotted fever seeds, including prototype strains, gave evidence of heterogeneity. We are currently examining this to determine which of our antigens might contain significant numbers of the extraneous agents and to determine if the prototype "strains" and the field isolates are indeed pure. This project has highest priority, since nothing else can proceed with any degree of confidence without the knowledge that the strains and serologic reagents prepared from them are indeed pure. This may in fact lead to a redefinition of some prototype strains which have been in rather wide use.

3. <u>Problems with the specific complement fixation test</u>. Over the years, we and others have had great difficulty in preparing highly purified rickettsial body antigens suitable for use in the CF test because as the antigens were purified, they tended to become "anticomplementary", regardless of which one of a wide variety of methods were used. This has seriously hampered the use of the specific CF test with the spotted fever group.

Recent preliminary work in our laboratory has suggested that conventional ether treated purified spotted fever group rickettsiae directly activate the C'3 component of the complement system in the absence of specific antibody. We have previously shown that typhus rickettsiae contain an endotoxin-like component and presume that spotted fever group rickettsiae possess an endotoxin-like component in the cell envelope which in the intact organism is covered by the "soluble" antigen in the external microcapsular layer. When the superficial layers are removed by aqueous-ether treatment as required to enhance "specificity" of the antigen, the endotoxin is exposed. The more complete the removal of surface group antigen, the more the endotoxin is exposed. The endotoxin activates C'3 directly through the alternate pathway, making the antigen "anticomplementary". Thus, the higher the degree of specificity of an antigen, i.e., the more efficient the removal of the group antigen, the more anticomplementary

the antigen becomes.

This paradoxical situation may limit the usefulness of the specific CF test unless alternate ways are found to control the exposure of the endotoxin. There may be practical ways of doing this.

4. **Specific Antisera.** Considerable difficulty has been encountered in the production of specific antisera for use in identification of spotted fever group rickettsiae. The result has been that guinea pigs have been recommended for some, mice for others, etc. We have some preliminary evidence to suggest that serum at a critical early stage after infection may exhibit a higher degree of specificity than is customarily experienced. We are exploring this to see if a battery of highly specific antisera can be prepared in this manner. If so, speciation would be greatly simplified.

We hope that this project will not only result in the clear-cut identification of new species and the improved characterization of recognized prototype species but also will provide a simpler, specific method for the identification of future isolates, so that the final acquisition of required information about an isolate can be accomplished in weeks rather then months to years.

E. **Scrub typhus.** As indicated in the renewal application for the past year, no further work has been done in the past year on the geographic distribution of serotypes, pending clarification of the status of the putative 5 new serotypes from Thailand by Elisberg et al, since it would be highly impractical to proceed with only 3 instead of possibly 8 prototypes. However, in the meantime, the plaquing technology has been developed to the state where plaque-purification of field isolates and their amplification for typing by the fluorescent antibody technique would be feasible on a routine basis.

Should the reagents for the 8 putative serotypes of R. tsutsugamushi not become available in a reasonable period of time, it will still be possible to test the major points if the alternative hypotheses related geographic distribution of serotypes, their genetic stability in nature and the presence or absence of unique geographic variants, although this will require some additonal effort over that which would be required if reagents for the most common types in epicenter of scrub typhus distribution were available.

F. **International Symposium on the Control of Lice and Louse-Borne Diseases.** As a direct result of our field studies in Burundi in 1969 on the E strain typhus vaccine, in which we incidentally recognized for the first time the widespread occurrence of human body lice resistant to malathion as well as DDT and related this most likely to the selective pressure incidental to the agricultural use of insecticides rather

than organized public health use, the responsible investigator
interested the Pan American Health Organization in holding an Inter-
national Symposium on the Control of Lice and Louse-borne Diseases in
Washington, D. C. on 4-6 Dec. 72. The symposium was jointly sponsored
by the Fogarty International Center, the U. S. Army Medical Research and
Development Command, the Pan American Health Organization and the
Commission on Rickettsial Diseases. The responsible investigator and
members of his staff played key roles in organizing and executing the
symposium. The Symposium covered a broad range of topics related to
the problems of lice and louse-borne diseases, discussed by an inter-
national group of experts. The proceedings have been published by the
Pan American Health Organization and constitutes the only modern,
comprehensive document on these problems.

G. Special Military Significance. The recent world-wide alert
of the U.S. Armed Forces in response to the Middle East crisis with
the attendant prospects of U.S. troops in the Middle East raised some
immediate problems with respect to rickettsial disease in the general
area. Known to be present are the following:

1. Louse-borne typhus. Epidemic during WW II, louse-borne
typhus has declined substantially in the village and urban populations
in recent years. However, outbreaks have occurred in recent years in
Beduin tribes as widely separated as Saudi Arabia and the Sahara Desert
in Southern Algeria. Moreover, an enormous human reservoir of typhus
exists both in the Arab population and in the Israeli population, the
latter being derived from European immigrants who experienced typhus
in WWII as well as immigrants from places such as Yemen, etc. Disorder
created by a more general conflict in that region would provide the
conditions for the resurgence of typhus.

2. Murine typhus. Murine typhus is known in Israel and
probably occurs in Egypt and other Arab countries as well. As shown
in Viet Nam, murine typhus has the potential for causing problems in
supply dumps and storage areas.

3. Q. fever. Q fever occurs in Israel. A high proportion
of people in Egypt have Q fever antibodies. The number of animals and
dust are conducive to spread of Q fever in these areas.

4. Tick-borne rickettsioses. R. conori is widespread
throughout the entire area, giving rise to classical fievre boutonneuse.
A more severe disease has been recognized in Israel and two probably
new species of the spotted fever group have been isolated. [We have
one of these strains for study and hope to get the other, as well.]

5. _Trench fever_. Antibodies to R. quintana are common in some areas of North Africa (Tunisia, for example - Vinson) and the disease is occurring in Burundi and Ethiopia. The morbidity due to this infection in North Africa, however, is unknown.

Of these diseases, only the louse-borne typhus and Q fever are vaccines potentially available for immunoprophylaxis. Other louse-borne diseases, i.e., Trench fever and relapsing fever, would not be prevented. Preparedness for the control of body lice in this region is not good. DDT resistance has long been known among body lice in Egypt. Resistance to lindane has been reported from the Sudan and quite likely exists in Egypt. Following our report of malathion resistance among body lice in Burundi, tests carried out in Egypt revealed malathion resistance there as well. Thus, resistance to all the standard insecticides has been found in body lice in Egypt. The situation in Syria, Jordan and other Arab countries of the area is unknown. It is clear, though, that substantial problems might be encountered in louse control programs which depend upon the acceptable insecticides readily available from military supply stocks.

The control of murine typhus would depend upon rodent and flea control. DDT resistance, at least, has been reported in _Xenopsylla cheopis_ fleas from Egypt.

APPENDIX I. Potential problem of selection of antibiotic resistant R. prowazeki under existing natural circumstances.

Louse-borne typhus is easily treated with chloramphenicol or the tetracycline series of antibiotics. Indeed, we have simplified the chemotherapy as a result of our studies in Burundi involving 250-300 patients so that we cure an adult typhus patient with a single 100 mg dose of doxycycline, a long-persisting tetracycline derivative, and have more limited observations to suggest that minocycline, another long-persisting tetracycline, will do the same. Moreover, it would be reasonable to predict that typhus-exposed men could be kept disease-free for substantial periods of time by as little as a single chemo-prophylactic dose of doxycycline per week. There is no doubt that a small typhus problem could be handled by chemotherapy/chemoprophylaxis alone, assuming (a) that the number of cases remained within that which could be spared from the operational load at the time and (b) that no antibiotic resistance was encountered. Unfortunately, factors beyond internal control actually operating in several parts of the world today have transformed the antibiotic resistance problem from a mere theoretical possibility to a probability which may have already occurred. The prospect of encountering an antibiotic-resistant typhus epidemic is very real. Very briefly, the salient observations and deductions which have led us to this conclusion are as follows: For convenience of presentation they can be divided into two categories, (a) the basic mechanisms and (b) the naturally occurring situations which permit the basic mechanisms to operate.

1) Basic mechanisms for development of resistance in R. prowazeki. Rickettsiae are now recognized as small bacteria which are obligate intracellular parasites. One might anticipate that their genetic mechanisms would be similar to those of other bacteria. Indeed, some years ago Weiss and his colleagues showed that typhus rickettsiae could be made antibiotic-resistant by serial passage in the yolk sacs of embryonated eggs in the presence of increasing drug concentrations. By techniques developed very recently in our laboratories through adaptations of the plaque technique and development of slide chamber technique, it is now possible to do quantitative studies on the action of antibiotics on typhus rickettsiae and it now appears to be quite feasible to measure mutation rates by the Luria-Delbruek fluctuation analysis with the same kind of precision that these have been measured with bacteria. Dose response curves to several antibiotics carried out essentially after Demerec, including doxycycline, have shown that the rickettsial population is very heterogeneous in sensitivity to antibiotics. For example, some R. prowazeki cells are inhibited by as little as 0.01 µg/ml of doxycycline; the majority are inhibited by 0.1 µg/ml, the effective minimal inhibitory concentration; but some

very preliminary tests have shown that about 0.05% of the cells are resistant to 1.0 ug/ml. It is anticipated that smaller numbers will be found resistant to 5-10 ug/ml and even higher. Thus, while we do not yet have an accurate mutation rate, the preliminary data strongly suggest that a sufficiently large population of R. prowazeki grown in the presence of doxycycline (or other antibiotics) could yield a population of significant resistance in "a single step". Two mechanisms by which these conditions could be met in nature exist: in the louse vector and in the human convalescent reservoir.

Recent studies in our laboratory on the louse vector have yielded two bits of quantitative information which bear on this problem. Direct counts have shown that infected body lice at the height of infection with virulent R. prowazeki harbor a population of 1-2 X 10^8 rickettsiae/louse (12). This enormous rickettsial population is more than adequate to have yielded mutants resistant to 10-fold or more the minimal inhibitory concentration of doxycycline. It has also been shown in our laboratory (12) that infected lice feeding upon blood containing therapeutically obtainable concentrations of doxycycline (or rifampin) ingest enough of the antibiotic to inhibit the growth of the bulk of the rickettsiae, thereby providing the required selective environment for the overgrowth of antibiotic-resistant strains of R. prowazeki. It remains to be demonstrated that such selection and overgrowth actually occur under conditions where infected body lice feed upon a subject receiving doxycycline therapy, but the quantitative relationships already in hand suggest that this could happen in a single louse passage. If transmitted to another person who survives the infection, a reservoir of antibiotic-resistant rickettsiae would be created.

The second mechanism by which resistant strains could arise involves the human reservoir of the rickettsiae, i.e., the person who has recovered from typhus. A non-sterile immunity follows recovery from epidemic typhus. The organisms may persist in the tissues in an unknown state for a very long time -- up to 20-30 years of more. At some time, perhaps many years, after the primary attack of typhus, some people experience a recrudescence of acute disease (Brill-Zinsser disease) in which the rickettsiae again circulate in the blood and are accessible to body lice, if present. Thus, a case of recrudescent typhus can serve to initiate a new louse-borne outbreak. Many millions of such reservoirs exist in all parts of the world today. In many of these places, even quite primitive areas, anti-biotics are readily available, often on the open market. It is likely that many typhus convalescents will take antibiotics from time to time over a period of 1-3 decades for a variety of other intercurrent infections. Indeed, chloramphenicol and tetracyclines are popular in primitive areas and are used freely when available. Thus, it is conceivable that selection of antibiotic-resistant strains could take place during the long period of inapparent infection and that some of these people would develop Brill-Zinsser disease at some time and that

transmission of the antibiotic-resistant strain could take place. Indeed, this could happen regardless if the antibiotic-resistance developed in the louse (as above) or in the human reservoir.

2) <u>Actual situation in which the selective mechanisms could operate and resistant strains could survive.</u> It is almost axiomatic that in places where typhus fever is occurring today, living conditions are primitive, medical care is limited and public health measures inadequate. On the basis of recent personal observations in Africa, it is a fact that more often than not the patient load is so great at bush hospitals and dispensaries, especially during a typhus outbreak, that neither typhus patients nor the other patients or family "nurses" with whom they are in intimate contact are deloused. With the simplified 1-dose doxycycline treatment, a typhus patient is simply given his capsule and remains in contact with the mass of humanity until he is afebrile and then leaves, with those infected lice which he has not already given to someone else. Since the doxycycline remains in the blood for several days, it (a) may provide the selective environment for the overgrowth of an antibiotic-resistant rickettsial population and (b) delays the death of the infected louse, thus increasing the possibility that such lice will be carried back to his community where transmission of now resistant rickettsiae might occur. Once in a new human host, the resistant strains can be perpetuated even though the louse dies. Paradoxically, subsequent to our demonstration that typhus could be cured by a single dose of doxycycline, WHO has been recommending its use even, and especially, in situations where simultaneous delousing of the patient is not possible on the assumption that the probable shortening of rickettsemia would reduce the period of infectivity of a patient. The information just related above has caused them some concern but the recommendation has not yet been rescinded and we have thus a recommendation still standing which inadvertently may contribute to the development of antibiotic-resistant typhus rickettsiae.

We believe that the mechanisms and actual situations are such that antibiotic-resistant strains -- to tetracyclines and perhaps chloramphenicol which also is widely used in the areas under consideration -- may have already been selected and established in reservoirs and that outbreaks of typhus caused by antibiotic-resistant strains are a real possibility for the future. It behooves us to investigate other antibiotics with suitable properties of effectiveness, availability, ease of administration, etc., which might not be used so frequently under the circumstances described to be held in reserve in the event that strains resistant to the more commonly used drugs are indeed encountered. For example, erythromycin inhibits the growth in cell culture and eggs of <u>R. prowazeki</u> in low

concentration (our unpublished studies in progress) and the growth of
R. mooseri and R. tsutsugamushi in somewhat higher concentrations (18),
has a therapeutic effect on scrub typhus, but is not useful for the
treatment of Rocky Mountain spotted fever. Rifampin inhibits the growth
of R. prowazeki in cell culture at a concentration of about 0.01 ug/ml.
These, and other antibiotics, warrant clinical evaluation in epidemic typhus.

III. PERSONNEL

The following persons contributed to the research reported in this document. As can readily be seen, only a portion of these were supported by the contract. This reflects the organization of much of the departmental research program in which multiple sources of support are focused on a limited array of problems. In this way a single program can serve several purposes which are mutually supportive - i.e., research and training. The training aspects, of course, are supported by an NIH training grant, but research done by these trainees contributes to the overall research program which is pertinent to this contract.

Section	Name	Rank	Source of Support Contract	Other

Responsible Investigator

	Charles L. Wisseman,Jr.,M.D.	Prof. and Head		+

Rickettsiology

Typhus Group

	George W. Sweeney	Lab. Scient. II		+
	Sarah C. Brown	Lab. Scient I	+	
	Frances M. Burnham	Lab. Tech		+
	B. LoVelle Beaman,M.A.	Grad. Fellow		+
	Laurie F. Gluck, B.A.	Grad. Fellow		+
	James R. Murphy	Grad. Fellow		+
	Denise D. Dalton, Ph.D.	Post-doctoral Fellow		+
	Agnes D. Waddell	Assistant	+	

Scrub Typhus Group

	William T. Walsh, M.S.	Assistant		+
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Metabolism Group

	William F. Myers, Ph.D.	Asst. Prof.		+
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Serology

	Paul Fiset,M.D.,Ph.D.	Assoc. Prof.		+
	Vernon W. Burnett, B.S.	Lab. Scient. I		+
	Marcia R. Schneider	Lab. Asst. II	+	

| Section | Name | Rank | Source of Support | |
			Contract	Other
Secretarial				
	Elizabeth P. Haviland	Office Sec. III		+
	D. Jean Wells	Office Sec. I		+
	E. Rebecca Alison	Clerk Typist IV P.T.	+	
Supportive				
	Lillian W. Parker	Lab. Asst. I		+
	Bernice Burton	Service Worker		+
	Leon Hawkins	Service Worker		+
	William W. Brown	Animal Attendt.	+	

IV. PUBLICATIONS UNDER CONTRACT IN PAST YEAR

1. Miller, R. N., C. L. Wisseman, Jr., G. W. Sweeney, A. Verschueren and I. B. Fabrikant. First report of resistance of human body lice to malathion. Trans. Roy. Soc. Trop. Med. Hyg. 66:372-375 (1972)

2. Wisseman, C. L., Jr. Concepts of louse-borne typhus control in developing countries: The use of the living attenuated E strain typhus vaccine in epidemic and endemic situations. In Kohn, A., and M.A. Klingberg (eds). Immunity in Viral and Rickettsial Diseases. Plenum Publishing Corp., N. Y., 1972, pp. 97-130.

3. Robertson, R. G. and C. L. Wisseman, Jr. Tick-borne rickettsiae of the spotted fever group in West Pakistan. II. Serological classification of isolates from West Pakistan and Thailand: evidence for two new species. Am. J. Epidemiol. 97:55-64 (1973)

4. Boese, J. L., C. L. Wisseman, Jr., and I. B. Fabrikant. Simple field method for disinfesting lice-infested clothing with dichlorvos strips. Trans. Roy. Soc. Trop. Med. Hyg. 66:950-953 (1972)

5. Boese, J. L., C. L. Wisseman, Jr., W. T. Walsh and P. Fiset. Antibody and antibiotic action on Rickettsia prowazeki across the cutaneous host-vector interface, with observations on strain virulence and retrieval mechanisms. Am. J. Epidemiol. 98:262-282 (1973).

6. Wisseman, C. L., Jr., D. W. Krause, I. B. Fabrikant and P. A. Mackowiak. Mechanisms of immunity in typhus infections. I. Action of human typhus convalescent serum on typhus rickettsiae in vivo and in vitro. J. Inf. Dis. (in press).

7. Gambrill, M. R., and C. L. Wisseman, Jr. Mechanisms of immunity in typhus infections. II. Multiplication of typhus rickettsiae in human macrophage cell cultures in the non-immune system: influence of virulence of rickettsial strains and of chloramphenicol. Infect. and Immun. 8:519-527 (1973).

8. Gambrill, M. R., and C. L. Wisseman, Jr. Mechanisms of immunity in typhus infections. III. Influence of human immune serum and complement on the fate of Rickettsia mooseri within human macrophages. Infect. and Immun. 8:631-640 (1973).

9. A series of papers and discussions in the <u>Proceedings of the
International Symposium on the Control of Lice and Louse-borne
Diseases</u> (4-6 Dec. 72), Scientific Publication No. 263, Pan
American Health Organization, Washington, D. C. (These will
not be listed individually.)   -

Manuscripts submitted for publication

1. Wisseman, C. L., Jr., A. D. Waddel and W. T. Walsh. Mechanisms
   of immunity in typhus infections. IV Failure of chick embryo
   cells in culture to restrict growth of antibody-sensitized
   <u>Rickettsia prowazeki</u>.

2. Wisseman, C. L., Jr., A. D. Waddell and W. T. Walsh. <u>In vitro</u>
   studies of antibiotic action on <u>Rickettsia prowazeki</u> by two
   basic cell culture methods.

Manuscripts in advanced stages of preparation

1. Wisseman, C. L. Jr., and A. D. Waddell. Infection cycle of
   <u>Rickettsia prowazeki</u> in cell culture. I. Kinetics of
   rickettsial uptake by chick embryo cells in slide chamber
   cultures and observations on mechanisms of entry.

2. Wisseman, C. L. Jr., and A. D. Waddell. Infection cycle of
   <u>Rickettsia prowazeki</u> in cell culture. II. One step growth
   cycle and growth kinetics of virulent and attenuated <u>R</u>.
   <u>prowazeki</u> in irradiated chick embryo cells in slide chamber
   cultures.

3. Wisseman, C. L. Jr., Dennis Brown, A. D. Waddell and W. T.
   Walsh. Kinetics and ultrastructure of unstable intracellular
   penicillin-induced spheroplast formation and disruption by
   <u>Rickettsia prowazeki</u>.

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